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Visualization by Immune Electron Microscopy of a 27-nm Particle Associated with Acute Infectious Nonbacterial Gastroenteritis

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A 27-nm particle was observed by immune electron microscopy in an infectious stool filtrate derived from an outbreak in Norwalk, Ohio, of acute infectious nonbacterial gastroenteritis. Both experimentally and naturally infected individuals developed serological evidence of infection; this along with other evidence suggested that the particle was the etiological agent of Norwalk gastroenteritis.

In spite of intensive efforts, an etiological agent has not been found for acute infectious nonbacterial gastroenteritis—a usually self-limited disease characterized by a spectrum of clinical symptoms which may include vomiting, diarrhea, abdominal pain, or a combination thereof (5, 10, 16). This syndrome affects a broad segment of the population and was the second most common disease experience in a 10-year family study (8, 9). The disease, which has been given various descriptive names, was transmitted to volunteers in the 1940's and 1950's and again more recently in 1971 and 1972, but all attempts to definitively cultivate and characterize an etiological agent in vitro have failed (5, 6, 11–17, 19, 21, 22). In the 1971 study, a filtrate from a rectal swab specimen from an adult who developed a secondary case of acute nonbacterial gastroenteritis during an outbreak in Norwalk, Ohio, induced the typical illness in two of three volunteers; it was serially passaged two additional times in volunteers and again induced the typical illness (1, 5, 11, 12). Characterization studies in volunteers revealed that the infectious agent in Norwalk outbreak-derived filtrates was less than 66 nm in diameter and probably less than 36 nm and, in addition, was not inactivated by ether, acid, or heating at 60 C for 30 min (11).

In an attempt to detect these fastidious, presumably viral, gastroenteritis agents, we adapted the technique of immune electron microscopy to the study of stool filtrates derived from the Norwalk outbreak. Previously, this method had been employed in serological or antigenic studies, or both, of various viruses, and was used for the successful observation of rubella virus (3, 4). Recently, this technique had facilitated the detection of Australia antigen and permitted the observation of rhinoviruses in semipurified suspensions (18, 23). Furthermore, immune electron microscopy has been employed successfully for the detection of an antigenic inner component of the Dane particle associated with hepatitis virus B and enabled the detection of a new coronavirus strain (2, 7, A. Z. Kapikian, H. D. James, Jr., S. J. Kelly, and A. L. Vaughn, Infect. Immunity **7**, 1973, *in press*). The present studies which resulted in the detection of small "picorna or parvovirus-like" particles to which certain volunteers and naturally infected individuals developed significant antibody increases are described below.

The 2% second human passage stool filtrate (8FIIa) used in these immune electron microscopy studies was derived from a stool specimen of a volunteer who developed gastroenteritis after oral administration of a stool filtrate derived from one of the two volunteers who became ill after receiving the original inoculum from the Norwalk outbreak (1, 11, 12). The 8FIIa pool, which had been filtered through a 1,200- and a 450-nm membrane filter (Millipore Corp.) and prepared by previously described methods, was known to contain an infectious agent; it had induced gastroenteritis in 6 of 10 volunteers, but extensive attempts to recover or detect an etiological agent by conventional techniques were unsuccessful (11, 12; R. Dolin et al., *unpublished studies*). Therefore, we examined this filtrate for the presence of virus particles by immune electron microscopy utilizing inactivated convalescent serum from experimentally infected volunteers as the source of

specific antibody as previously described in our coronavirus studies (A. Kapikian et al., Infect. Immunity **7**, 1973, *in press*). This approach was taken in the hope that virus particles would appear in the form of aggregates, thereby enabling the observation of a small virus, possibly present in low titer. The serum-stool filtrate mixtures (and at various times, 0.85% phosphate-buffered [*p*H 7.4] saline [PBS]-stool filtrate mixtures) were incubated at room temperature for 1 hr routinely. PBS was then added, if necessary, to make a final pre-centrifugation volume of 1.0 ml for each mixture. The mixtures were then centrifuged at 17,000 rev/min for 90 min in a Sorvall RC2B centrifuge with an SS34 fixed-angle rotor. The supernatant fluid was carefully discarded; the pellet or sediment was suspended with a few drops of distilled water, stained with 3% phosphotungstic acid (PTA), *p*H 7.2, placed on a 400-mesh Formvar-carbon coated grid, with the excess fluid being removed with the edge of a filter paper disc, and the grid examined at a magnification of 40,000 with a Siemens Elmiskop 1A electron microscope (3, 18).

In the initial experiment, reaction of 0.4 ml of the 8FIIa stool filtrate with 0.1 ml of a 1:10 dilution of convalescent serum from volunteer A who developed the typical illness after challenge with an 8FII stool filtrate (*see* Table 1) resulted in the appearance of aggregates similar to the one shown in Fig. 1. The particles which were heavily coated with antibody were not randomly distributed but were present as groups which stood out clearly from the surrounding matter and appeared to resemble the picorna or parvoviruses.

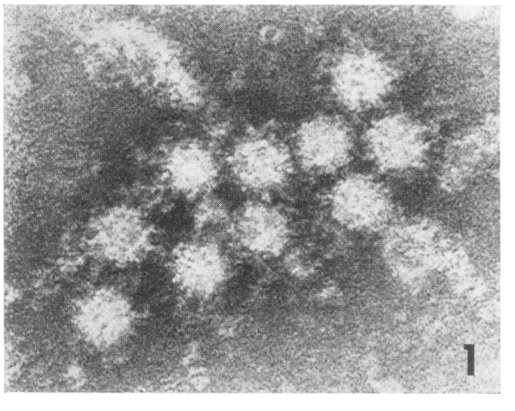

FIG. 1. *An aggregate observed after incubation for 1.75 hr of the stool filtrate with a 1:10 dilution of postchallenge antiserum of volunteer A in the second experiment. The particles which are heavily coated with antibody were not randomly distributed but were present mostly as aggregates which stood out clearly from the surrounding matter.* ×*231,500.*

In stool filtrate-PBS control preparations, occasional particles or groups of particles without apparent antibody were seen, and Fig. 2 shows a particularly favorable orientation of six such particles. The significance of these particles would have been difficult to evaluate without the previous experience acquired from examining similar, but heavily coated, particles which had been aggregated by antibody. These particles appeared to have cubic symmetry and there was a suggestion of surface substructure, but a definite pattern could not be ascertained (Fig. 2). They measured approximately 27 nm in their shortest diameter and 32 nm in their longest and again resembled both the picornaviruses and the parvoviruses morphologically.

These studies were then extended to include both prechallenge and convalescent sera in an attempt to detect serological evidence of infection by immune electron microscopy. A 0.2-ml amount of a 1:5 dilution of uninactivated serum was mixed with 0.8 ml of the 8FIIa stool filtrate since in preliminary studies 0.1 ml of a higher dilution of serum plus 0.4 ml of the 8FIIa stool filtrate resulted in variable staining with PTA. In these serological studies, the grids were read without prior knowledge of the specimen being examined in order to eliminate the possibility of biased interpretation. Routinely, five squares on each grid were examined in a median time of approximately 1 hr, and the preparation was then rated for the quantity of antibody as follows: 0 = no aggregates (3 or more particles in a group were considered to constitute an aggregate); 1+ = glistening aggregates, lightly covered with antibody; 2+ = moderately glistening aggregates, moderately covered with antibody; 3+ = non-

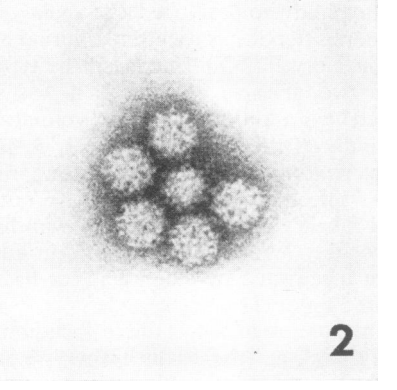

FIG. 2. *A group of particles observed after incubation of the stool filtrate with PBS in the initial experiment. This group of particles without apparent antibody appeared to have cubic symmetry, and there was a suggestion of surface substructure, but a definite pattern could not be ascertained.* ×*231,500.*

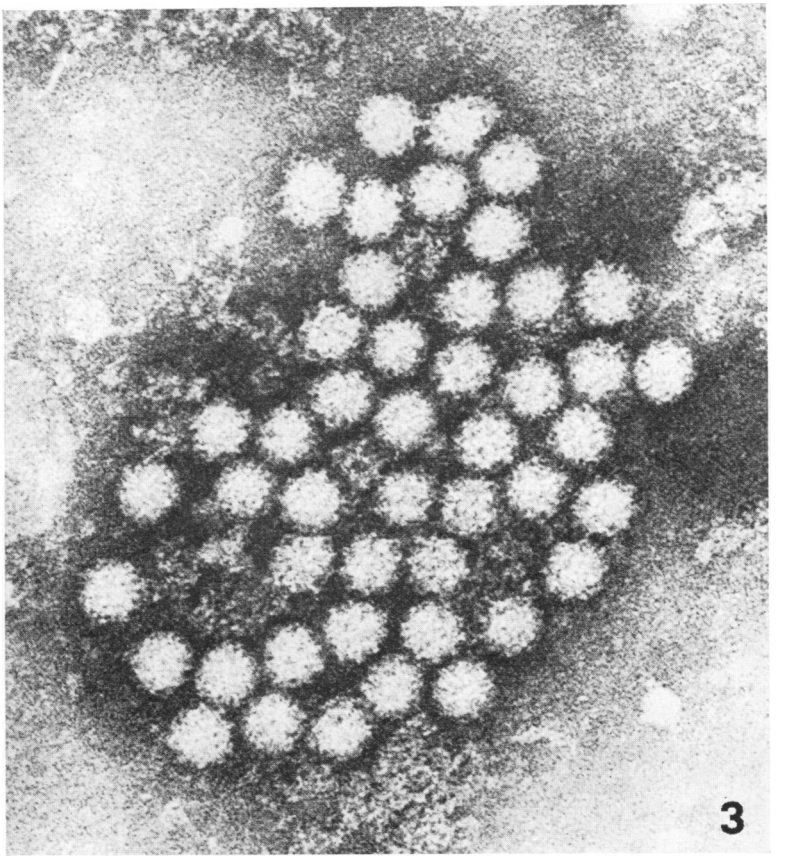

FIG. 3. *An aggregate observed after incubation of the stool filtrate with a 1:5 dilution of prechallenge antiserum of volunteer A. The quantity of antibody on these glistening particles was scored as 1+. ×231,500.*

glistening aggregates, heavily coated with antibody; 4+ = nonglistening aggregates so heavily coated with antibody that they were almost obscured. A 1+ difference was considered to be a significant change in the amount of antibody present. An example of an aggregate scored as 1+ is shown in Fig. 3, and another scored as 4+ is shown in Fig. 4.

The paired sera from volunteer A were tested as noted above with the 8FIIa stool filtrate. The pre-illness serum mixture yielded one aggregate which was lightly covered with antibody, whereas with the convalescent serum specimen approximately 15 very heavily coated aggregates were observed. As shown in Table 1, these pre- and postsera were rated as 1+ and 4+, respectively; no aggregates were observed in a PBS control in which 0.2 ml of PBS was incubated with 0.8 ml of the 8FIIa stool filtrate.

Volunteer B, who developed the typical experimental illness, also demonstrated an increase in antibody by immune electron microscopy from 2+ to 4+ with 4 and 10 aggregates, respectively (Table 1). Similar increases in antibody were seen with volunteers C and D who also became ill after challenge with the 8FIIa stool filtrate, whereas volunteer E, who did not develop illness, did not have a significant change in antibody titer (Fig. 5 and 6, and Tables 1 and 2). No aggregates were observed in PBS-8FIIa stool filtrate mixtures.

Although all four volunteers who developed illness after challenge with the 8FIIa stool filtrate developed serological evidence of infection, it was possible that the observed particles might represent a virus not related to the etiological agent of the Norwalk outbreak; it was conceivable that an adventitious virus could either have been present in the stool of the patient from the original Norwalk outbreak or could have been acquired during passage through volunteers. We examined this possibility by testing paired sera from six individuals from the Norwalk outbreak; four were primary cases, one a secondary case and one a contact who did not become ill (1). Serum sam-

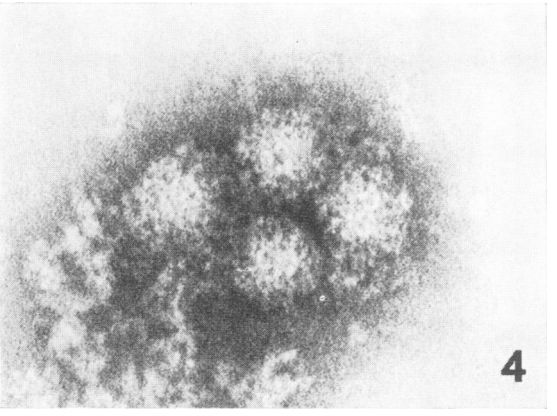

FIG. 4. *An aggregate observed after incubation of the stool filtrate with a 1:5 dilution of postchallenge antiserum of volunteer B. The particles were not glistening and were so heavily coated with antibody that they were almost obscured. The quantity of antibody on these particles was scored as 4+. Heavily coated particles were usually found to be in small aggregates, whereas those with less antibody usually formed larger aggregates.* ×*231,500.*

TABLE 1. *Serologic evidence of infection detected by immune electron microscopy: study of volunteers with experimentally induced illness and individuals in original Norwalk outbreak*[a]

Category	Inoculum	Individual	Gastrointestinal illness	Serum	Amount of antibody present on particles[b]	No. of aggregates observed in five squares
Experimentally infected volunteers	Second passage stool filtrate (8FII)[c] derived from stool of individual L below	A	Yes	Prechallenge	1	1
				Postchallenge	4	ca. 15
		B	Yes	Prechallenge	2	4
				Postchallenge	4	10
		C	Yes	Prechallenge	1–2	3
				Postchallenge	4	3[d]
		D	Yes	Prechallenge	2–3	4[e]
				Postchallenge	4	4
		E	No	Prechallenge	1	3
				Postchallenge	1–2	2
Naturally occurring illness—Norwalk, Ohio, outbreak		F[f]	Yes	Acute	1–2	4
				Convalescent	3	15
		G[f]	Yes	Acute	4	6
				Convalescent	4	8
		H[f]	Yes	Acute	2	5
				Convalescent	4	6
		I[f]	Yes	Acute	3–4	8
				Convalescent	3–4	6
		J[g]	Yes	Acute	<1	3
				Convalescent	2	9
Contact—Norwalk, Ohio, outbreak		K	No	Early	1	7
				Late	0	0
Experimentally infected volunteers	Filtrate from rectal swab from individual J above	L	Yes	Prechallenge	2–3	7
				Postchallenge	3–4	3
		M	Yes	Prechallenge	1	4
				Postchallenge	0[d]	

[a] In experiments with sera from individuals A, B, C, D, E, F, and K, 8FIIa incubated also with PBS as control and no aggregates were observed.
[b] After incubation with individual serum.
[c] 8FII and 8FIIa derived from stools obtained from same individual during same illness.
[d] Six squares counted.
[e] Eight squares counted.
[f] Primary case.
[g] Secondary case.

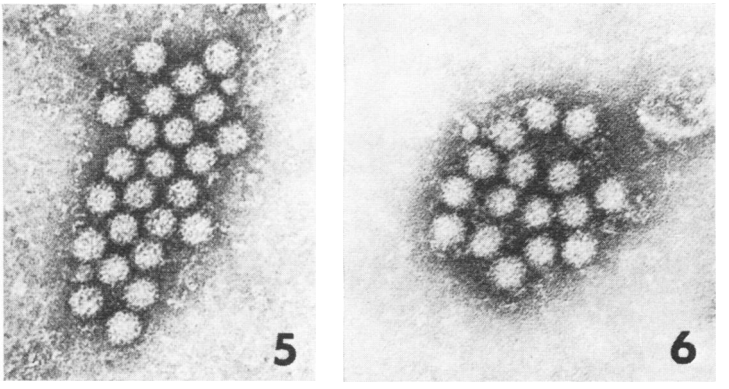

FIG. 5 and 6. *Aggregates observed after incubation of the stool filtrate with a 1:5 dilution of prechallenge antiserum (Fig. 5) and a 1:5 dilution of postchallenge antiserum (Fig. 6) of volunteer E who did not become ill after challenge. No significant change in quantity of antibody on the particles was evident (prechallenge serum rated 1+; postchallenge, 1-2+). The glistening quality of the lightly coated aggregates is readily evident at this lower magnification also (cf. Fig. 3). ×138,900.*

TABLE 2. *Serological evidence of infection detected by immune electron microscopy: study of sequentially challenged volunteers*[a]

Serum sequence tested (prechallenge [indicated by Pre] and days postchallenge [indicated by number])			Estimated quantity of antibody associated with aggregates of 27-nm particles[b]	
1st Challenge	2nd Challenge	3rd Challenge	Volunteer no. C	Volunteer no. D
Pre			2	2[c]
27			1–2	3
52	4		1	2–3[d]
83	35	Pre	1–2[e]	2–3[c]
104	56	21–25	4[f]	4

[a] In these experiments, 8FIIa stool filtrate incubated also with PBS as control and no aggregates were observed.

[b] Volunteers C and D developed gastroenteritis after first and third challenges. Volunteer C challenge sequence: H filtrate, H filtrate, 8FIIa filtrate. Volunteer D challenge sequence: MC filtrate, MC filtrate, 8FIIa filtrate. H and MC filtrates each derived from a secondary case of gastroenteritis in Honolulu (H), Hawaii, and Montgomery County (MC), Md., respectively.

[c] Eight squares counted.

[d] Nine squares counted.

[e] Plasma utilized since serum not available.

[f] Six squares counted.

ples were kindly supplied by Milford H. Hatch, Jonathan L. Adler, and Raymond Zickl. As shown in Table 1, three of the five persons with naturally acquired Norwalk gastroenteritis developed an increase in antibody to the 27-nm particles during convalescence from disease, whereas the two who did not show such an increase demonstrated a high degree of antibody in both their acute and convalescent sera. The acute-phase sera were not collected until several days after the onset of disease, and this may explain our failure to detect a response in two of the patients. The contact who did not become ill similarly did not exhibit a serum response (Table 1); one of the aggregates observed with the contact's early serum was comprised not only of the usual "full" particles but with two "empties" as well (Fig. 7). It is of interest that among the seroresponders was the donor (J) of the original rectal swab specimen which had induced illness in two of three volunteers in the 1971 study (1, 12). We examined by immune electron microscopy the prechallenge and convalescent sera of these two volunteers (L and M) and found that one developed an increase in antibody whereas the other did not (Table 1). The development of an increase in antibody by three of the five naturally ill individuals suggests that the 27-nm particle was not acquired during passage in volunteers. Furthermore, these serum responses suggest that the virus-like particles had infected patients during the Norwalk outbreak.

Finally, we investigated the possibility that gastrointestinal disease might stimulate the observed antibody responses by a nonspecific mechanism. In another study to be reported in detail later, volunteers were first administered either a stool filtrate containing an infectious agent from a secondary case of gastroenteritis which had occurred in Honolulu, Hawaii, or from a secondary case which had occurred in Montgomery County, Md., (R. G. Wyatt et al., *unpublished studies*). Those volunteers who became ill after primary challenge were later rechallenged with the same agent, and homologous immunity was demonstrated. Subsequently, challenge with a filtrate derived from the Norwalk outbreak produced

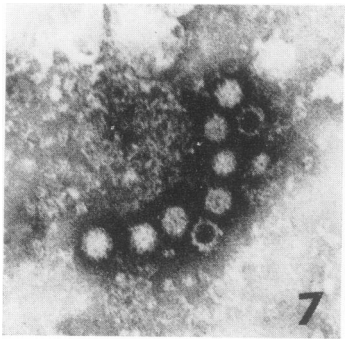

FIG. 7. *An aggregate observed after incubation of the stool filtrate with a 1:5 dilution of early serum from contact K. This small aggregate (scored 1+) was comprised not only of the usual "full" particles but contained two "empties" as well.* × *138,900.*

disease, indicating that the filtrates derived from the Hawaii and Maryland cases differed from that of Norwalk. Sequential sera from two volunteers (C and D described previously) who underwent the sequence of three challenges just described were studied by immune electron microscopy with the 8FIIa pool as antigen. The volunteer administered the filtrate derived from the Hawaii case failed to develop an increase in antibody following gastroenteritis induced by the primary challenge, but did develop a significant response after illness induced by the Norwalk filtrate, suggesting that the immune electron microscopy seroresponse was specific (Table 2). The other volunteer developed an increase in antibody after gastroenteritis induced by primary challenge and a further increase after illness induced by the Norwalk filtrate, suggesting that the infectious agents in the Maryland and the Norwalk filtrates may have been antigenically related (Table 2).

It was noteworthy that each of the 13 individuals demonstrated the presence of antibody in pre-, acute-phase, or early sera, suggesting that infection with the agent derived from the Norwalk outbreak, or a related agent (or agents), was quite common. Possibly, the agents of nonbacterial gastroenteritis may resemble certain respiratory viruses in their capacity to reinfect with facility.

We have presented data suggesting that the 27-nm particle was the etiological agent of Norwalk gastroenteritis. Although it is conceivable that the 27-nm particle induced infection which was not related to the disease, it is unlikely. In any case, additional laboratory and epidemiological studies are needed to confirm the postulated etiological relationship.

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